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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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SERIAL NO. :   09/950,003   ART UNIT : 1623

FILED   :   September 12, 2001   CONFIRM NO.: 9777

FOR   GLYCOSAMINOGLYCANS DERIVED FROM K5
POLYSACCHARIDE HAVING HIGH ANTICOAGULANT
AND ANTITHROMBOTIC ACTIVITIES AND PROCESS
FOR THEIR PREPARATION

May 13, 2008

THIRD PARTY OBSERVATION ON PRIOR ART
RELEVANT TO APPLICATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

1) Applications

The application 09/950,003, publication number US 2002/0062019,
which have the filing date 12.9.2001, with title: "glycosaminoglycans derived fom K5 polysaccharide having high anticoagulant and antithrombotic activities and process for their preparation".
This documents claims as priority the Italian application IT MI2000A000665 of 30 Mar 2000

The application 11/030,156, publication number US 2005/0215518, divisional application of the above one, which have the filing date 7.1.2005, with title: "glycosaminoglycans derived fom K5 polysaccharide having high anticoagulant and antithrombotic activities and process for their preparation".
This documents claims as priority the Italian application IT MI2000A000665 of 30 Mar 2000

2) Claims

Part of the claims of the above applications concerns the use and the condition of use of the enzyme glucuronosyl C5 epimerase in presence of divalent cations. These contents are reported in claims 20, 21 as currently pending in US 09/950,003, and its Divisional US 11/030,156, claim 4.

3) Prior art

The prior art described in the above documents and cited during the present examination did not include the following documents:

1) Jacobsson I et al . *J. Biol Chem.* Vol. 254. No. 8, Issue of April 25, pp. 2975-2982, 1979.
Title: Biosynthesis of Heparin , ASSAY AND PROPERTIES OF THE MICROSOMAL URONOSYL C-5 EPIMERASE (Received for publication, March 20, 1978) Printed in U.S.A
Autors:
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David S. Feingold, From the Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261
Anders Malmström and Lennart Rodén, From the Institute of Dental Research and Department of Medicine, University of Alabama in Birmingham, Birmingham, Alabama 35294 '

2) Malmstrom A et al. *J. Biol Chem.* Vol. 255. No. 9. Issue of May 10, pp. 3878-3883, 1980.
Title: Biosynthesis of Heparin, PARTIAL PURIFICATION OF THE URONOSYL C-5 EPIMERASE (Received for publication, September 20, 1979) Printed in U.S.A
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These two publications describe the use of bivalent ions for the activity of the enzyme Glucuronosyl C5 epimerase:

In particular **document n°1** describes in the chapter "RESULTS" at pag 2976 that : *No specific metal ion requirement was observed, but addition of salts, including EDTA, NaCl, MgCl2, and CaCl2 markedly stimulated the reaction over a rather narrow concentration range (Fig. 5). It may be noted that in contrast to the other salts tested, MnCl2 failed to stimulate the enzyme.*
The cited Fig 5 in the text reports the effect of various cations on uronosyl C-5 epimerase activity in the presence of NaCl, EDTA, $MgCl_2$, $MnCl_2$ or $CaCl_2$ in a range of concentration between 0 and 200 mM

Document n° 2 reports in "RESULTS AND DISCUSSION" chapter at pag 3880 the following description:
The enzyme was active over a narrow pH range, with an optimum at pH 7.4 (Fig. 6). Particularly important from a practical point of view was the marked dependence on ionic strength. As seen in Table I, the enzyme had negligible activity in 0.05 M Hepes, 0.05 M KCl, pH 7.4, but addition of EDTA to a final concentration of 0.015 M (standard buffer) yielded maximal tritium release. Similar results were obtained when the KCl concentration was increased to 0.1 M or upon addition of other salts (Na2SO4, Na2HPO4, and MgCl2) at appropriately chosen concentrations.

These two publications clearly anticipate the art described by the application EP 1358215 and their cited priorities appl US 09/738,879 and appl US 09/950,003.

Annex: 1) Jacobsson I et al . J. Biol Chem. Vol. 254. No. 8, Issue of April 25, pp. 2975-2982, 1979.
2) Malmstrom A et al. J. Biol Chem. Vol. 255. No. 9. Issue of May 10, pp. 3878-3883, 1980.

Biosynthesis of Heparin

ASSAY AND PROPERTIES OF THE MICROSOMAL URONOSYL C-5 EPIMERASE*

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It was previously shown that the formation of L-iduronic acid residues by C-5 epimerization of D-glucuronic acid units at the polymer level during the synthesis of heparin involves exchange of the hydrogen atom at C-5 with protons of the medium (Lindahl, U., Jacobsson, I., Höök, M., Bäckström, G., and Feingold, D. S. (1976) *Biochem. Biophys. Res. Commun.* 70, 492-499). When a heparin precursor polysaccharide composed of alternating D-[5-³H]glucuronosyl and N-sulfated D-glucosaminosyl residues was incubated with a microsomal mouse mastocytoma preparation, the ³H located on C-5 was exchanged with protons of the medium. The aqueous portion of the reaction mixture was isolated by low temperature distillation, and the ³H present was determined by liquid scintillation counting. The rate of ³H release was directly proportional to the concentration of microsomal enzyme as well as to substrate concentration and was used as an assay for the uronosyl 5-epimerase. The reaction had a pH optimum close to 7.4. No requirement for specific metal ions was observed; however, the reaction rate was strongly dependent on the ionic strength of the medium.

Incubation of various 5-³H-labeled heparin precursor polysaccharides showed that only N-sulfated preparations were substrates; the best substrate consisted largely of alternating D-glucuronosyl and N-sulfated D-glucosaminosyl moieties. Exhaustive incubation of this substrate with microsomal enzyme caused release of 60 to 70% of the ³H originally present; concomitantly, the L-iduronic acid content increased from 18 to 29% of the total uronic acid. When the reaction mixture was supplemented with 3'-phosphoadenylylsulfate, more than half of the substrate was converted into an O-sulfated species which contained 41% L-iduronic acid. These results suggest that although exchange of the C-5 hydrogen atom always accompanies 5-epimerization, the converse does not always occur.

In contrast to the exogenous substrate, the membrane-bound endogenous intermediates did not lose tritium in excess of the extent of conversion of D-glucuronic acid to L-iduronic acid. Apparently, the high degree of organization in the native biosynthetic system permits a stricter regulation such that each attack by the epimerase is carried through to inversion of configuration.

Earlier investigations of the biosynthesis of heparin have defined the basic features of this process as it occurs in the microsomal fraction of a transplantable mouse mastocytoma. Endogenous substrates present in this cell fraction serve as primers for polysaccharide chain elongation, and following the formation of a nonsulfated polymer composed of alternating D-glucuronic acid and N-acetyl-D-glucosamine units (4–6), a series of extensive modifications take place which complete the biosynthetic events and yield the biologically active product. These modifications, which occur in a stepwise manner, include N-deacetylation, N-sulfation,[1] C-5 epimerization of more than half of the uronic acid residues, and finally, O-sulfation in two positions (1, 3, 7, 8). Structures of the intermediates formed during this process are shown in Fig. 1.[2]

The mechanisms of the polymer modification reactions are poorly understood; also, the manner in which the cell achieves operational coordination between the various enzymes is as yet completely unknown. A major difficulty in approaching these problems has been the lack of specific, exogenous substrates, which has, in particular, limited the scope of investigations of the epimerase. However, it was recently found that formation of the L-iduronic acid units is accompanied by

[1] The abbreviations used are: N-sulfate, sulfamino group; O-sulfate, ester sulfate group. See Fig. 1 (miniprint supplement) for abbreviations of polysaccharides which are intermediates in heparin biosynthesis.

[2] Portions of this paper (including "Materials and Methods," additional references, Figs. 1 and 2, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-458, cite author(s), and include a check or money order for $1.65 per set of photocopies.

exchange of the hydrogen at C-5 of the D-glucuronic acid precursor residues (2). This observation forms the basis for an assay of the epimerase in which release of tritium from an enzymatically synthesized, 5-³H-labeled heparin precursor is measured. This approach has also enabled us to determine certain basic properties of the epimerase, and the results of this work are described in this report.

METHODS

Assay of Uronosyl C-5 Epimerase—Reaction mixtures contained the following components in a total volume of 0.25 ml: 0.05 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, pH 7.4; 25 mM EDTA; tritium-labeled polysaccharide substrate (500 to 2000 cpm of [5-³H]PS-NSO₃⁻ or else as specified in the text); and enzyme (0.25 mg or less of microsomal protein). After incubation at 37°C for 30 min, 0.05 ml of water was added, and the reaction was stopped by heating at 100°C for 2 min. The samples were transferred to round bottom tubes with ground glass joints (Kontes K-897900) and were rapidly frozen in a dry ice/ethanol mixture. The sample tube and a receiving tube (Kontes K-410050) were then connected to a distillation apparatus, as illustrated in Fig. 3. The system was evacuated with a Sargent-Welch Duo-Seal pump, model No. 1400, and was disconnected from the pump while still under vacuum. Distillation was begun by heating the sample in a water bath at 30°C while the receiving tube was cooled in dry ice/ethanol, and was usually completed in 5 to 10 min. Aliquots of the distillate (0.2 ml) were subjected to scintillation counting. To conserve substrate, relatively small amounts were used for each assay point, which often necessitated a counting time of 20 min. For values below 100 cpm, the standard error of duplicate assays was ±2.2 cpm (54 observations), and in the range of 100 to 600 cpm (19 observations), the standard error was ±7.8 cpm.

RESULTS

Tritium Release from [5-³H]PS-NSO₃⁻—Incubation of the N-sulfated, D-[5-³H]glucuronosyl-labeled heparin precursor ([5-³H]PS-NSO₃⁻) with mast cell microsomes resulted in release of the radioactivity as tritiated water. The reaction was linear for at least 1 h and reached a plateau when 60 to 70%
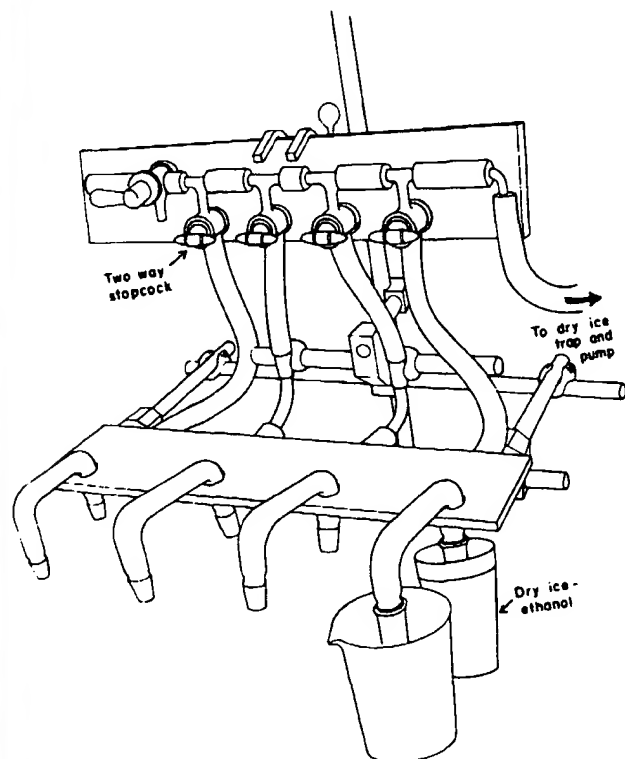

FIG. 3. Distillation device used to recover tritiated water after incubation of labeled polysaccharide substrates with microsomal uronosyl C-5 epimerase.
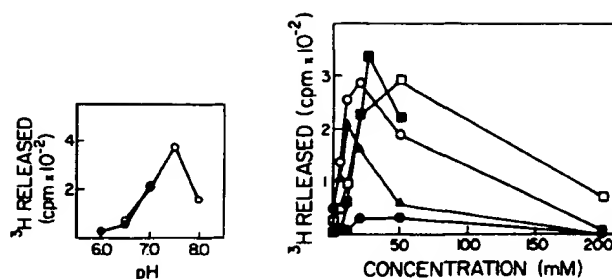

FIG. 4 (*left*). Effect of pH on uronosyl C-5 epimerase activity. Microsomal enzyme was incubated with tritium-labeled substrate ([5-³H]PS-NSO₃⁻; 1700 cpm) under standard conditions (30 min; 1 mg of protein/ml; see "Methods"), with 0.05 M 2-(N-morpholino)ethanesulfonic acid (●——●) or 0.05 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (○——○) as buffer.

FIG. 5 (*right*). Effect of various cations on uronosyl C-5 epimerase activity. Microsomal enzyme was incubated with tritium-labeled substrate ([5-³H]PS-NSO₃⁻) in the presence of NaCl (□——□), EDTA (■——■), MgCl₂ (○——○), MnCl₂ (●——●), or CaCl₂ (▲——▲). Incubations were for 30 min with 1100 cpm of substrate at a protein concentration of 1 mg/ml.

of the tritium had been liberated.[3] The release of radioactivity was also directly proportional to enzyme concentration within the range tested (up to 1 mg of protein/ml). The pH optimum was around 7.5 (Fig. 4). No specific metal ion requirement was observed, but addition of salts, including EDTA, NaCl, MgCl₂, and CaCl₂ markedly stimulated the reaction over a rather narrow concentration range (Fig. 5). It may be noted that in contrast to the other salts tested, MnCl₂ failed to stimulate the enzyme.

The K_m of the substrate was 2×10^{-8} M, expressed as concentration of D-glucuronosyl residues. This remarkably low value is subject to revision, however, since the actual concentration of substrate (exogenous radioactive plus endogenous unlabeled) was not known and the calculation of K_m was based on the specific activity of the UDP-D-[5-³H]glucuronic acid used in the preparation of the exogenous substrate.

Characterization of Reaction Products—To determine the relationship between loss of hydrogen and conversion of D-glucuronic to L-iduronic acid residues, PS-NSO₃⁻ doubly labeled in the D-glucuronic acid component with ¹⁴C and ³H (at C-5) was incubated with microsomal enzyme as described in detail in the legend to Fig. 6. After 1 h, approximately 60% of the tritium in the substrate had been released, and no further loss occurred upon reisolation of the polysaccharide and repeated incubation with the enzyme. Of the residual ³H label, 27% was eliminated as tritiated water by digestion with chondroitinase ABC (see also Footnote 3). The [¹⁴C]uronic acid composition of the final product, which was devoid of ³H label susceptible to epimerase or to chondroitinase ABC, was determined; a control reaction mixture incubated with heat-inactivated epimerase and active chondroitinase ABC was similarly analyzed. In four separate experiments, an average increase in L-iduronic acid content from 18 to 29% was observed.[4] Since this increase of 11% corresponds to less than 20% of the tritium release and, in addition, the remaining D-

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[3] About 30% of the remaining label resided in chondroitin sulfate, as demonstrated by digestion with chondroitinase ABC (9) followed by gel chromatography.

[4] The respective values for control and incubated material were: 17 and 29%; 18 and 29%; 18 and 29%; and 21 and 29%. The two latter sets of values were obtained from experiments in which 10 mM MnCl₂, 10 mM MgCl₂, and 5 mM CaCl₂ were included in the reaction mixtures instead of 25 mM EDTA. Due to inadequate analytical procedures, the presence of L-iduronic acid in PS-NSO₃⁻ was overlooked in previous analysis of this material (1).

glucuronic acid units had a ^{3}H/^{14}C ratio only about half that of the starting material, some of the D-glucuronic acid residues must have lost their C-5 tritium atoms, yet retained the D-*gluco* configuration.

The final reaction product was further characterized by treatment with nitrous acid at pH 1.5 and subsequent gel chromatography on Sephadex G-25 (Fig. 6). As judged by the elution pattern of the ^{14}C label, the deamination products comprised approximately 70% disaccharides and 20% higher oligosaccharides. The approximately 10% of the deamination products which emerged at the void volume presumably represents N-acetylated block sections of the parent precursor polysaccharide. This fraction contained virtually all the ^{3}H label remaining after incubation with the epimerase and had a ^{3}H/^{14}C ratio close to that of the corresponding material from a control incubation with heat-treated enzyme. In contrast, the disaccharides derived from substrate which had been exposed to active epimerase contained 5% or less of the ^{3}H label found in the control material. These results demonstrate that the 5-epimerase labilizes the C-5 ^{3}H atoms of the D-glucuronosyl moieties in the N-sulfated regions of the heparin precursor molecule but has no effect on those D-glucuronosyl moieties present in the N-acetylated block sections.

Substrate Specificity—Some information concerning the mechanism of epimerase action and a more precise delineation of the structural features necessary for release of the C-5
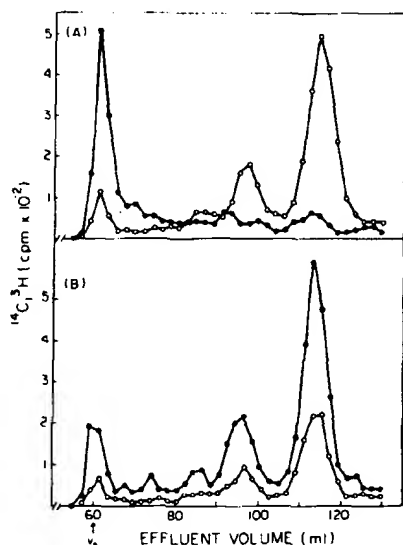

FIG. 6. Gel chromatography on Sephadex G-25 of products obtained by deamination of doubly labeled epimerase substrate ([^{14}C, 5-^{3}H]PS-NSO$_3^-$) after incubation with (A) active microsomal enzyme and (B) heat-inactivated microsomal enzyme. Samples of [^{14}C, 5-^{3}H]PS-NSO$_3^-$ (1.3 × 10^5 cpm of ^{3}H; 5 × 10^4 cpm of ^{14}C) were incubated at 37°C with active or heat-inactivated microsomal protein (8.0 mg) in 4.0 ml of 0.05 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, pH 7.4, containing 25 mM EDTA. More enzyme (8.0 mg) was added after 1 h, and incubation was continued for a total of 3 h. The course of ^{3}H release was measured on 0.02-ml aliquots which were removed at zero time and at hourly intervals. After completed incubation, the polysaccharide products were recovered by digestion with papain followed by gel chromatography and desalting (see "Materials and Methods"). They were then treated with chondroitinase ABC (9) and reisolated by gel chromatography on Sephadex G-50. Part of the excluded material was analyzed for uronic acid composition. The remainder of *Sample A* and half of *Sample B* were treated with 0.2 ml of nitrous acid reagent at pH 1.5 (10) for 10 min. After interruption of the deamination reaction by the addition of 0.025 ml of 2 M Na$_2$CO$_3$ the samples were applied to a column (1 × 185 cm) of Sephadex G-25, equilibrated with 0.2 M NaCl. The column was eluted at a rate of 4 ml/h; effluent fractions of 2 ml were collected and analyzed for ^{3}H (●——●) or ^{14}C (○——○).

TABLE II

Tritium release from ^{3}H-labeled polysaccharides by microsomal uronosyl C-5 epimerase

Polysaccharide substrate	^{3}H released[a]
	cpm
[5-^{3}H]PS-NAc	<20
[5-^{3}H]PS-NH$_3^+$	<20
[5-^{3}H]PS-NH$_3^+$, N-acetylated	<20
[5-^{3}H]PS-NH$_3^+$, N-deacetylated and N-sulfated	188
[5-^{3}H]PS-NSO$_3^-$	640
[5-^{3}H]PS-NSO$_3^-$ (heat-inactivated control)	<20
[5-^{3}H]PS-NSO$_3^-$, N-desulfated	<20
[5-^{3}H]PS-NSO$_3^-$, N-desulfated and re-N-sulfated	114
[5-^{3}H]PS-N/O-SO$_3^-$	252
[4-^{3}H]PS-NSO$_3^-$	<20
[3-^{3}H]PS-NSO$_3^-$	<20
[2-^{3}H]PS-NSO$_3^-$	31
[5-^{3}H]Chondroitin	<20

[a] Samples (2000 cpm of ^{3}H) were incubated according to the standard procedure (see "Methods"); 0.25 mg of microsomal protein was added per incubation (0.25 ml). The amounts of tritium released could be increased further by prolonging the incubation time.

hydrogen was obtained by the following experiments. As shown in Table II, loss of tritium occurred specifically from the C-5 position, since little or no radioactivity was released from the 2-, 3-, and 4-labeled analogs of [5-^{3}H]PS-NSO$_3^-$. In addition to [5-^{3}H]PS-NSO$_3^-$, the O-sulfated heparin precursor, [5-^{3}H]PS-N/O-SO$_3^-$, was also a substrate, but the nonsulfated polysaccharides, [5-^{3}H]PS-NAc and [5-^{3}H]PS-NH$_3^+$, were not substrates, suggesting that N-sulfation is a prerequisite to 5-epimerization. The substrate activity of [5-^{3}H]PS-NSO$_3^-$ was abolished by chemical desulfation and partially restored by re-N-sulfation; likewise, N-sulfation converted [5-^{3}H]PS-NH$_3^+$ into a substrate.[5] These findings further attest to the specific need for N-sulfate groups in the substrate structure. It should also be noted that no tritium release occurred from [5-^{3}H]chondroitin.

A similar picture emerged from additional experiments in which unlabeled polysaccharides and polysaccharide derivatives were incubated with the enzyme together with the labeled substrate, [5-^{3}H]PS-NSO$_3^-$ (Table III). Heparin, a potent inhibitor of tritium release, lost most of its inhibitory activity on desulfation (combined with N-deacetylation); the activity was restored by N-sulfation but not by N-acetylation. The low-sulfated heparan sulfate from human aorta, itself a poor inhibitor, was converted into a highly efficient inhibitor by N-deacetylation (not sufficient for activation) followed by N-sulfation. Neither chondroitin sulfate, dermatan sulfate, nor hyaluronic acid showed significant inhibitory activity.

Effect of O-Sulfation on Epimerization—A close relationship between O-sulfation and the extent of uronic acid epimerization was previously demonstrated in studies of heparin biosynthesis from endogenous precursors (1). In the present investigation, a similar relationship was shown using exogenous substrate. A sample of [^{14}C]PS-NSO$_3^-$ (uniformly labeled uronic acid residues; ratio of [^{14}C]iduronic acid to total [^{14}C]-uronic acid, 0.20) was incubated with mastocytoma microsomal enzyme in the absence or presence of 3'-phosphoaden-

[5] In order to provide more sites for N-sulfation, the polysaccharide, [5-^{3}H]PS-NH$_3^+$ (having about half of its glucosamine residues N-acetylated; see Table I) was chemically N-deacetylated prior to the reaction with trimethylamine·sulfur trioxide complex. The final product was a substrate for the epimerase but never acquired the same substrate activity as [5-^{3}H]PS-NSO$_3^-$ (Table II). This discrepancy may be due to incomplete N-sulfation (Table I) but also to the introduction of some O-sulfate groups (L.-Å. Fransson, personal communication) leading to the formation of polymer sequences not recognized as substrate by the epimerase.

ylylsulfate (for details, see legend to Fig. 7). After isolation from the reaction mixture, the labeled polysaccharide was analyzed by ion exchange chromatography on DEAE-cellulose (Fig. 7). The sample which had been incubated in the absence

TABLE III

Effect of unlabeled polysaccharides on tritium release from
([5-³H]PS-NSO₃⁻)

Unlabeled polysaccharide added	³H released[a]	
	μg	%
None		100
Heparin	25	9
	100	4
Heparin, desulfated and N-deacetylated	25	92
	100	91
Heparin, desulfated and N-acetylated	25	78
	100	80
Heparin, desulfated, N-deacetylated, and N-sulfated	25	24
	100	2
Heparan sulfate	25	83
	100	64
Heparan sulfate, N-deacetylated	25	80
	100	71
Heparan sulfate, N-deacetylated, and N-sulfated	25	2
	100	1
Dermatan sulfate	25	88
	100	91
Chondroitin sulfate	25	97
	100	88
Hyaluronic acid	25	92
	100	90

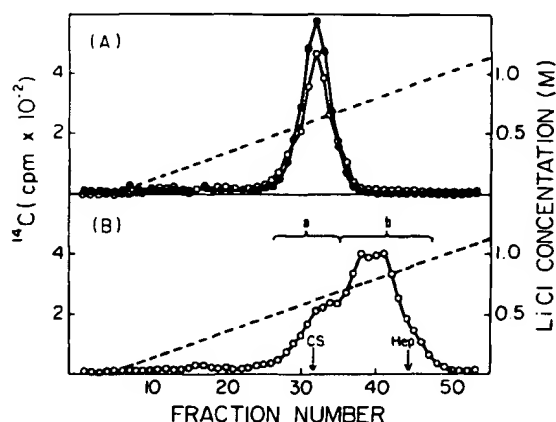[a] Percentage of label released under standard incubation conditions in the absence of unlabeled polysaccharide.

FIG. 7. Chromatography on DEAE-cellulose of ¹⁴C-labeled epimerase substrate ([¹⁴C]PS-NSO₃⁻) after incubation with microsomal enzyme in the absence (A) or presence (B) of 3'-phosphoadenylylsulfate. O——O, sample incubated with active enzyme; ●——●, control with heat-inactivated enzyme (the control has been omitted from B but was essentially identical with that shown in A). Labeled polysaccharide (2 × 10⁴ cpm of ¹⁴C) was dissolved in 1.0 ml of 0.05 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, pH 7.4, containing 10 mM MnCl₂, 10 mM MgCl₂, and 5 mM CaCl₂; to the appropriate samples were added 2.0 μmol of 3'-phosphoadenylylsulfate. After the addition of 10 mg of microsomal protein, the samples were incubated at 37°C for a total period of 3 h, with repeated addition of 1.0 μmol of 3'-phosphoadenylylsulfate after 1 h. The polysaccharides were reisolated after papain digestion by gel chromatography on Sephadex G-50 and were then desalted, as described under "Materials and Methods." The products were subjected to ion exchange chromatography on DEAE-cellulose (DE52) as described under "Materials and Methods." Radioactive effluent fractions were pooled and desalted; the material incubated with active enzyme in the presence of 3'-phosphoadenylylsulfate was recovered in two fractions, a and b, as indicated. The *arrows* indicate the peak elution positions of standard preparations of chondroitin sulfate (*CS*) and heparin (*Hep*). ‐‐‐, concentration of LiCl in the elution gradient.

of 3'-phosphoadenylylsulfate emerged as a sharp, symmetrical peak in the same position as the heat-inactivated control (Fig. 7A). In contrast, a major portion of the material which had been incubated in the presence of 3'-phosphoadenylylsulfate appeared in a more retarded position (Fraction b in Fig. 7B), suggesting that the substrate had been modified by the introduction of O-sulfate groups. The presence of such groups was verified by paper electrophoresis (1) of disaccharides obtained by nitrous acid deamination of Fraction b at pH 1.5 followed by reduction with sodium borohydride. Monosulfated uronosylanhydromannitol constituted 28% of the ¹⁴C activity of Fraction b; however, no di-O-sulfated disaccharide was detected. In addition to the O-sulfated material (Fraction b), a fraction was also recovered which appeared to have escaped O-sulfation during the incubation with 3'-phosphoadenylylsulfate (Fraction a in Fig. 7B). No significant amounts of O-sulfated species were present in the starting material, [¹⁴C]PS-NSO₃⁻.

Determination of the uronic acid composition of the various fractions showed that incubation of PS-NSO₃⁻ in the absence of 3'-phosphoadenylylsulfate increased the L-[¹⁴C]iduronic acid content from 20 to 29%, in accord with data presented earlier. Fraction a had a similar content of L-iduronic acid (27%). In the O-sulfated Fraction b, however, L-iduronic acid constituted 41% of the total uronic acid. These results suggest that whereas N-sulfation of the heparin polysaccharide precursor is the only prerequisite for conversion of D-glucuronic acid to L-iduronic acid residues, O-sulfation enhances the extent of the 5-epimerization.

Relationship between ³H Release and Epimerization; Role of Exogenous or Endogenous Substrate—In a previous communication (2), in which the loss of tritium which accompanies conversion of D-[5-³H]glucuronosyl to L-iduronosyl moieties was first reported, examination of the ³H/¹⁴C ratio of microsome-bound intermediates (PS-NAc, PS-NH₃⁺, PS-NSO₃⁻, and PS-N/O-SO₃⁻ (endogenous substrates)) showed that the tritium release paralleled the extent of formation of L-iduronosyl moieties. It was tacitly assumed that there was no loss of tritium from D-glucuronosyl residues which were not converted to the L-iduronosyl structure; however, the validity of this assumption was not tested. Since it has now become apparent that in exogenous substrates exchange of C-5 hydrogen atoms on D-glucuronosyl moieties exceeds the extent of their conversion to L-iduronosyl residues, the fate of the C-5 tritium atoms of the D-glucuronosyl moieties of the endogenous substrate was re-examined.

TABLE IV

Analysis of polymeric intermediates formed by incubation of mastocytoma microsomes with UDP-[¹⁴C]glucuronic acid, UDP-[5-³H]glucuronic acid, UDP-N-acetylglucosamine, and 3'-phosphoadenylylsulfate

Preparation[a]	[¹⁴C]Iduronic acid content[b]	³H/¹⁴C ratio of glucuronic acid residues[c]
	%	
PS-NAc + PS-NH₃⁺	<5	1.00
PS-NSO₃⁻	19	0.93
PS-N/O-SO₃⁻	44	1.03

[a] For procedures used in preparing the various intermediates, see "Materials and Methods." All fractions were derived from the same preparation of labeled microsomal polysaccharide; the components PS-NAc and PS-NH₃⁺ (recovered combined into one fraction) represent material that remained nonsulfated. After degradation of polysaccharides, the resulting uronic acid monosaccharides were separated by paper chromatography and analyzed for ³H and ¹⁴C.

[b] Expressed as percentage of total [¹⁴C]uronic acid ([¹⁴C]iduronic acid plus [¹⁴C]glucuronic acid).

[c] The ³H/¹⁴C ratio for the glucuronic acid recovered from PS-NAc + PS-NH₃⁺ was arbitrarily set as 1.00.

In the experiment described in Table IV, doubly labeled polysaccharides were prepared by incubation of microsomal enzyme with UDP-D-[^{14}C]glucuronic acid, UDP-D-[5-^{3}H]glucuronic acid, UDP-N-acetyl-D-glucosamine, and 3'-phosphoadenylylsulfate. After completed incubation, the products were separated into three fractions (PS-NAc + PS-NH$_3$$^+$, PS-NSO$_3$$^-$, and PS-N/O-SO$_3$$^-$), and the D-glucuronic acid component from each was isolated and analyzed with regard to its ^{3}H/^{14}C ratio. As seen from Table IV, this ratio was essentially the same for all fractions despite the difference in conversion of D-glucuronosyl to L-iduronosyl structures.

DISCUSSION

The conversion of D-glucuronosyl to L-iduronosyl moieties which occurs in the biosynthesis of heparin and dermatan sulfate is the only polymer level carbohydrate epimerization known in mammals. In the analogous 5-epimerization of D-mannuronosyl to L-guluronosyl moieties during alginic acid synthesis by *Azotobacter vinelandii* (11, 12), ^{3}H was incorporated from ^{3}H$_2$O into the L-guluronosyl moieties (13), presumably at C-5. Labilization of the C-5 hydrogen atom thus seems to be a common feature of 5-epimerization reactions.

The observed loss of the C-5 ^{3}H during the 5-epimerization of the 5-^{3}H-labeled heparin precursor, PS-NSO$_3$$^-$, is consistent with a reaction mechanism in which the C-5 hydrogen atom of the reactive uronic acid moiety is exchanged with protons of the medium. The reaction can be considered to involve at least two identifiable steps, either of which could be rate-limiting: 1) abstraction of a proton from C-5 of the D-glucuronosyl moiety to yield an intermediate (possibly a carbanion), and 2) addition of a proton from the medium to this intermediate, in either the D-glucuronosyl or L-iduronosyl configuration. (Chemically N-deacetylated or N-sulfated heparan sulfate incorporates label equally into D-glucuronosyl and L-iduronosyl moieties when incubated with microsomal enzyme in ^{3}H$_2$O,[6] confirming involvement in the reaction of an intermediate which exchanges protons with the medium.) This proton exchange is confined to C-5, since PS-NSO$_3$$^-$ labeled at C-2, C-3, or C-4 did not release ^{3}H when incubated with the enzyme. ^{3}H is released from the precursor substrate as a linear function of time and enzyme concentration; also, the release has a pH optimum and displays saturation kinetics, all of which are in accord with accepted criteria of enzyme action. The initial rate of proton loss can thus be used as a reliable measure of 5-epimerase activity, even though it may not indicate the actual extent of epimerization. The simple, rapid, and reproducible assay procedure described here permits study of the epimerization without interference from subsequent reactions involved in heparin biosynthesis, such as O-sulfation.

When exogenous 5-^{3}H-labeled PS-NSO$_3$$^-$ is exhaustively treated with enzyme, loss of C-5 ^{3}H from reactive D-glucuronosyl moieties is essentially complete and clearly exceeds the extent of conversion of D-glucuronosyl to L-iduronosyl moieties. These findings suggest that under the experimental conditions chosen, the 5-epimerization had reached equilibrium. Although the data do not permit an accurate determination of an equilibrium constant, the ratio of L-iduronosyl to D-glucuronosyl moieties at equilibrium appears to be approximately 1:1.[7]

In contrast to the extensive loss of ^{3}H observed with exogenous PS-NSO$_3$$^-$, there is a strict proportionality between loss of ^{3}H and conversion of D-glucuronosyl to L-iduronosyl moieties when endogenous substrate is processed by the enzyme (Table IV). This difference may reflect the highly organized nature of the reaction sequence in the endogenous system, in which not only the polymer-modifying enzymes but presumably also their polysaccharide substrates are bound to the membranes of the microsomal preparation. As a result, only a fraction of the D-glucuronosyl residues are attacked by the epimerase and, furthermore, each attack is carried through to complete inversion of configuration at C-5, *i.e.* to formation of an L-iduronic acid unit. It was shown in previous studies with endogenous substrates that formation of L-iduronosyl moieties occurs along with O-sulfation (1, 7); it is therefore possible that the introduction of O-sulfate groups impedes recognition of D-glucuronosyl moieties in the vicinity as substrates for the epimerase. However, additional selection mechanisms must be involved, since the O-sulfated product, [5-^{3}H]PS-N/O-SO$_3$$^-$, was shown to contain a significant proportion of reactive [5-^{3}H]glucuronosyl moieties when added as an exogenous substrate to the microsomal enzyme (Table II).

The studies of substrate specificity summarized in Table II show that only N-sulfated polymers of appropriate structure are substrates for the enzyme. In keeping with its presumed position in the sequence of heparin biosynthesis, PS-NSO$_3$$^-$ is the most effective of all substrates tested.[8] Confirmation of the indispensable role of N-sulfation is provided by examination of the ability of unlabeled polymers to inhibit ^{3}H release from [5-^{3}H]PS-NSO$_3$$^-$ (Table III). Inhibition of ^{3}H release by heparin does not necessarily indicate that this polysaccharide can serve as a substrate for the 5-epimerase, but rather may mean that it binds to and inhibits the enzyme by virtue of its similarity to the substrate.

The role of O-sulfation in relation to uronosyl C-5 epimerization is more difficult to define. As already mentioned, previous studies with endogenous substrates showed that formation of L-iduronosyl moieties is somehow linked to O-sulfation (1, 7); furthermore, L-iduronic acid units and O-sulfate groups are accumulated within the same sections of the heparin chain (14). These results were tentatively explained by postulating preferential O-sulfation of L-iduronic acid-containing polymer sequences, leading to the formation of structures that are not substrates for the 5-epimerase and therefore are withdrawn from the D-glucuronosyl-L-iduronosyl equilibrium. Such a mechanism is compatible with the results obtained in the present study on incubating exogenous PS-NSO$_3$$^-$ with microsomal enzyme in the presence of 3'-phos-

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[6] I. Jacobsson and U. Lindahl, unpublished results.

[7] In calculating this ratio (actual value, 3:4), it is assumed that all the L-iduronic acid (*i.e.* about 30% of the total ^{14}C-labeled uronic acid of the incubation product) but only part of the D-glucuronic acid units (about 40% of the total [^{14}C]uronic acid) are involved in the equili-

bration; the remaining D-[^{14}C]glucuronic acid residues occur in N-acetylated regions of the substrate, inaccessible to the epimerase, and in chondroitin (see the text).

[8] The data available do not permit any conclusions as to whether two successive D-glucosamine units have to be N-sulfated in order to render the interjacent D-glucuronic acid unit a substrate for the epimerase. In a previous study, L-iduronic acid residues were found in internal positions of hexa- and octasaccharides isolated after deaminative cleavage of heparin with nitrous acid; it was concluded that the neighboring D-glucosamine residues were N-acetylated and hence that N-sulfation of these residues was not a prerequisite to uronic acid C-5 epimerization (7). However, it has since been demonstrated that the deamination conditions employed at that time do not effect quantitative cleavage of N-sulfated D-glucosamine residues (10); in fact, renewed treatment of the same oligosaccharide fraction with nitrous acid, under more adequate conditions (aqueous pH 1.5 procedure described by Shively and Conrad (10)), led to appreciable degradation of the material. Some or all of the internal L-iduronic acid residues may thus have been located in juxtaposition to N-sulfated D-glucosamine units.

phoadenylylsulfate. However, it cannot fully account for the analogous modification of endogenous substrate. As already discussed, C-5 epimerization of the endogenous polysaccharide never attains equilibrium but, in fact, appears to be irreversible. The precise interrelation between epimerization and O-sulfation in the native biosynthetic system thus remains elusive and, at present, it can only be stated with certainty that the two processes are strongly interconnected.

The biosynthesis of dermatan sulfate resembles that of heparin in that the formation of L-iduronic acid residues is associated with O-sulfation of the molecule (15). However, the unique requirement for N-sulfate groups in the substrate of the mastocytoma epimerase indicates that two different epimerases are involved in the biosynthesis of heparin and dermatan sulfate. In keeping with this notion, chondroitin was not a substrate for the mastocytoma epimerase, nor did chondroitin sulfate and dermatan sulfate inhibit this enzyme.

A problem of nomenclature has become apparent in the course of these and previous studies on heparin biosynthesis, *i.e.* the lack of a suitable name for the D-glucuronic acid-containing polysaccharide which forms the backbone of the intermediates, PS-NAc and PS-NH$_3^+$ (and most of PS-NSO$_3^-$). To facilitate the description of some of these substances, we propose the name "heparosan" for the polysaccharide composed of D-glucuronic acid and D-glucosamine, in which the glucuronidic linkages are β1,4 and the glucosaminidic linkages are α1,4. The primary biosynthetic product (PS-NAc) would then be "N-acetylheparosan." With the qualification that the polymer modification reactions may result in considerable structural heterogeneity, we might use the name "heparosan N-sulfate" for PS-NSO$_3^-$. Accordingly, the enzyme described here should be called "heparosan N-sulfate

D-glucuronosyl 5-epimerase." This name may not be ideal but has certain advantages, at least in oral presentation, over the rational name, poly[(1 → 4)-β-D-glucopyranosyluronic acid-(1 → 4)-2-deoxy-2-sulfoamino-α-D-glucopyranosyl] glucopyranosyluronic acid 5-epimerase.

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MATERIALS AND METHODS

Materials

Unlabeled UDP-N-acetyl-D-glucosamine was obtained from Sigma Chemical Co., St. Louis, Mo. UDP-N-Acetyl-D-galactosamine was synthesized according to Roseman et al. (1). UDP-D-[^{14}C]glucuronic acid (uniformly labeled; 321 Ci/mol) was purchased from the Radiochemical Centre, Amersham, U.K. 3'-Phosphoadenylylsulfate was prepared using enzymes from rat liver high-speed supernatant as described (2,3), except that ammonium bicarbonate (nonlinear gradient from 0.014 M to 0.7 M) was used to elute the DEAE-cellulose column. Chondroitinase ABC was obtained from Miles Laboratories, Elkhart, Indiana.

UDP-D-glucuronic acid labeled with ^{3}H in the D-glucuronosyl moiety was prepared as follows. D-[^{3}H]Glucose labeled with ^{3}H at C-2 (10^4 Ci/mol), C-3 (2.1 x 10^3 Ci/mol), C-4 (6.1 x 10^3 Ci/mol) or C-5 (2.4 x 10^3 Ci/mol) was purchased from Amersham-Searle, Chicago, Ill. All enzymes and other materials used in the preparation were the purest available and purchased from Sigma Chemical Co. with the exception of UDP-D-glucose dehydrogenase, which was purified to homogeneity as described by Zalitis and Feingold (4). Reaction mixtures contained the following: a) reagents (mM): ATP, 0.9; phosphoenolpyruvate, 3.6; UTP, 1.2; MgCl$_2$, 0.8; D-glucose 1,6-diphosphate, 0.01; NAD, 1.6; cysteine, 40; Tris-HCl, pH 7.5, 28.5; and b) enzymes (international units/ml): hexokinase, 1.7; inorganic pyrophosphatase, 2.8; pyruvate kinase, 3.5; phosphoglucomutase (activated by incubation in 0.27 M cysteine (5)), 2.5; UDP-D-glucose pyrophosphorylase, 0.6; and UDP-D-glucose dehydrogenase, 0.3, in a total volume of 3.5 ml. The reaction was started by addition of the labeled D-glucose dissolved in 100 μl of water. The reaction mixture was incubated at 25° for 15 h and finally held at 100° for 2 min. After centrifugation the supernatant fluid was treated with 5 international units of E. coli alkaline phosphatase at 25° for 3 h. Salts were removed by passage through a 4.5 x 45 cm column of Biogel P-2 which was eluted with distilled water at 4°. Radioactive fractions were pooled, concentrated at 25°, retreated with alkaline phosphatase, and the radioactive material was desalted on a Biogel column as described. Final purification of the UDP-D-[^{3}H]glucuronic acid was achieved by paper chromatography in 95% ethanol-1 M ammonium acetate, pH 7.5 (7:3, v/v). The radioactive material, which had the mobility of authentic UDP-D-glucuronic acid, was eluted with water, concentrated to dryness in vacuum at 25° over KOH and H$_2$SO$_4$, and finally dissolved in water to the desired concentration.

Oligosaccharides obtained by degradation of heparin with nitrous acid were prepared as described previously (6). Samples of hyaluronic acid and chondroitin 4-sulfate, isolated from rooster combs and from bovine nasal septa, respectively, were generous gifts from Dr. A. Wasteson, University of Uppsala, Sweden. Dermatan sulfate was prepared from heparin side fractions by alkaline copper precipitation (7); residual heparin-like polysaccharides were eliminated by deaminative cleavage with nitrous acid followed by dialysis. Low-sulfated heparan sulfate was isolated from human aorta, as described by Iverius (8). Heparin (stage 14, isolated from pig intestinal mucosa), obtained from Inolex Pharmaceutical Div., Park Forest South, Ill., U.S.A., was purified by repeated precipitation with cetylpyridinium chloride from 1.2 M NaCl, essentially as described (7).

Radioactively labeled microsomal heparin-precursor polysaccharides were prepared by incubating mixtures containing, per ml of 0.05 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid buffer, pH 7.4; labeled UDP-D-glucuronic acid (either 25 μCi UDP-D-[^{3}H]glucuronic acid, 10 μCi UDP-D-[^{14}C]glucuronic acid, or a mixture of 25 μCi UDP-D-[^{3}H]glucuronic acid and 2.5 μCi UDP-D-[^{14}C]glucuronic acid); 2.5 μmol UDP-N-acetyl-D-glucosamine; mouse mastocytoma microsomal fraction, corresponding to 10 mg protein; 10 μmol MnCl$_2$; 10 μmol MgCl$_2$; and 5 μmol CaCl$_2$. After incubation at 37° for 3 h, the mixtures were digested with papain (9), and labeled polysaccharide was isolated by gel chromatography on columns (1 x 90 cm) of Sephadex G-50 eluted with 1 M NaCl. The polysaccharides emerged as distinct peaks of radioactivity at the void volume; they were desalted either by dialysis or by passage through a column of Sephadex G-25, eluted with 10% aqueous ethanol. No carrier polysaccharide was added. The yield of polysaccharide ranged between 3 x 10^5 and 5 x 10^5 cpm ^{3}H per ml of incubation mixture. Sulfated polysaccharide was prepared in a similar manner, except that 3'-phosphoadenylylsulfate (1 μmol per ml incubation mixture) was added twice during the incubation, at the start and after 60 min.

Labeled heparin precursor polysaccharide preparations were fractionated by anion-exchange chromatography on DEAE-cellulose as described (10), with the exception that the column was operated at room temperature rather than at 60°. Elution was performed with the aid of LKB model 11300 Ultrograd gradient mixer; gradients from 0.05 M to 0.75 M LiCl and from 0.05 M to 1.5 M LiCl were employed in the separation of nonsulfated and sulfated preparations, respectively. By this procedure the nonsulfated polysaccharide was fractionated into two components, PS-NH$_2$ and PS-NAc (corresponding to Fractions I and II, respectively, in Fig. 1, Ref. 10); the sulfated materials yielded in addition, PS-NSO$_3^-$ and PS-N/O-SO$_3^-$ (Fractions III and IV, respectively, in Fig. 1, Ref. 10; see also Fig. 3, Ref 11). The characteristic structural features of the various components are shown in Fig. 1. The isolated fractions were desalted and stored at -70°.
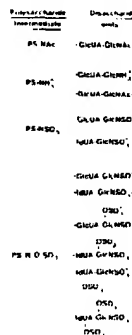

Fig. 1. Representative disaccharide units of microsomal polymeric intermediates. In addition to the N-sulfated glucosamine residues shown in the figure, PS-NSO$_3^-$ and PS-N/O-SO$_3^-$ also contain small amounts of N-acetylated glucosamine units. The occurrence of L-iduronic acid (10 to 20% of the total uronic acid) in PS-NSO$_3^-$ was not recognized in a previous study (10) but has since been clearly demonstrated by more refined analysis (see Methods). The various disaccharide units in PS-N/O-SO$_3^-$ were identified after deaminative cleavage of the polysaccharide and separation of the resulting disaccharides by paper electrophoresis and paper chromatography (I. Jacobsson, M. Höök, I. Pettersson, U. Lindahl, O. Larm and K. v. Figura, unpublished observations). The intermediates PS-NAc, PS-NH$_2$, PS-NSO$_3^-$, and PS-N/O-SO$_3^-$, mentioned in order of formation during heparin biosynthesis, correspond to the chromatographic fractions II, I, III and IV, respectively, described in a previous publication (10). Fractions I to III appear fairly homogeneous on ion-exchange chromatography, whereas fraction IV (PS-N/O-SO$_3^-$) is markedly heterogeneous.

Some of the labeled and unlabeled polysaccharide preparations were subjected to various chemical modification procedures (see below for methods). Analytical data for the resulting products as well as for the parent polysaccharides are given in Table I.

Chondroitin containing 5-^{3}H-labeled D-glucuronic acid residues was prepared using UDP-D-[5-^{3}H]glucuronic acid in a reaction mixture identical to that used to prepare heparin precursors (see Ref. 12), except that 3'-phosphoadenylylsulfate was omitted and UDP-N-acetyl-D-galactosamine was substituted for UDP-N-acetyl-D-glucosamine. The labeled product was eluted in the same position as hyaluronic acid in DEAE-cellulose chromatography (10), and all its radioactivity was released as ^{3}H$_2$O on digestion with chondroitinase ABC (13). Although not rigorously identified as such, the labeled material thus appears to be chondroitin.[1]

Microsomal fraction from mouse mastocytoma was prepared by homogenization of the tissue in 4 volumes of 0.25 M sucrose, followed by centrifugation at 20,000 x g for 20 min; the supernatant fraction was centrifuged at 100,000 x g for 60 min, and the resulting pellet was suspended in 0.25 M sucrose.

Analytical Methods

Methods for the determination of protein and radioactivity have been described (11). Uronic acid was determined by the method of Bitter and Muir (14), with D-glucuronolactone as standard. Hexosamine analyses were performed by a modification (15) of the Elson-Morgan reaction, after hydrolysis of saccharides in 4 M HCl at 100° for 14 h. Ratios of D-glucosamine to D-galactosamine were measured after separation on a column of Aminex A-5 (Bio-Rad Laboratories, Richmond, Calif.), as described by Lohmander (16). Sulfate was determined by the method of Terho and Hartiala (17). Ratios of unlabeled D-glucuronic acid to L-iduronic acid were determined by gas-liquid chromatography after degradation of polysaccharides and conversion of the resulting uronic acid monosaccharides into the corresponding aldono-1,4-lactones (6). Ratios of D-[^{14}C]glucuronic acid to L-[^{14}C]iduronic acid were determined essentially as described (6,18), with the following slight modification. After chromatographic separation of the monosaccharides obtained by degradation of the polysaccharides by acid hydrolysis and deamination with nitrous acid, the entire paper strip was cut into 1-cm segments, and the radioactivity present in each was determined by scintillation counting. This technique is more accurate than the previous method in which only the areas of paper containing the labeled uronic acids (located with a strip scanner) were excised and counted.

The N-substitution pattern of polysaccharides was determined by gel chromatography of the products obtained by selective deamination with nitrous acid. In this process susceptible D-glucosamine residues are converted to 2,5-anhydro-D-mannose units, with cleavage of the corresponding glucosaminidic linkages. Samples of each polysaccharide were treated with nitrous acid at pH 4.3 (Reaction B in Ref. 9), leading to deamination of only those D-glucosamine residues in which the amino groups were unsubstituted (19). The products were separated by gel chromatography on Sephadex G-25. Another set of samples was subjected to two successive deamination treatments in which the first deamination at pH 4.3 was followed by deamination at pH 1.5. In the latter reaction attack occurs at the N-sulfated, but not the N-acetylated residues present in fragments resulting from the first deamination (19). The size-distribution of the twice-deaminated material was determined by gel chromatography on Sephadex G-25. The individual effluent fractions of the various chromatographic runs were grouped and classified according to the elution position of standard heparin oligosaccharides as di-, tetra-, hexa- and octasaccharides and larger, excluded material. The proportions of N-unsubstituted, N-sulfated and N-acetylated D-glucosamine residues in the intact polysaccharides were calculated from the relative amounts of the various oligosaccharides obtained by deamination; it was assumed that whereas deamination products larger than disaccharide might contain N-sulfated as well as N-acetylated D-glucosamine residues after the first deamination, only N-acetylated units would be present after the second (pH 1.5) deamination. Obviously, the experimental error in this method may be considerable, and the results (Table I), although satisfactory for the purposes of the present study should be regarded as approximations. Experimental details are given in the legend to Fig. 2, showing the gel chromatograms obtained after deamination of native and modified heparan sulfate.

Chemical Modification of Polysaccharides

Complete desulfation of polysaccharides was carried out by the method of Kantor and Schubert (20) with the modification that the total reaction time was 6 or 9 days; the acetylchloride-methanol mixture was changed after each 3-day period. Selective N-desulfation was performed by the method of Nagasawa and Inoue (21). N-Deacetylation was achieved by treatment of polysaccharides with hydrazine in the presence of hydrazine sulfate (22) at 100° for 2 h. Polysaccharides with N-unsubstituted D-glucosamine residues were N-acetylated with acetic anhydride (23) or N-sulfated by reacting with trimethylamine-sulfurtrioxide complex (24). Complete details of these procedures will be described elsewhere.

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[1] Since the chondroitinase is a hexosaminidase acting by β-elimination mechanism, the resulting disaccharides have Δ4,5-unsaturated uronic acid residues from which the hydrogens, originally in 5-position, have been released. Gel chromatography of the digestion products on Sephadex G-15 showed a ^{3}H-labeled component that was retarded in relation to Δ4,5-unsaturated reference disaccharides. Removal of water by distillation depleted the ^{3}H-labeled fractions of all radioactivity.

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Table I Analysis of polysaccharide preparations

Preparation	Uronic acid[b]	Hexosamine[b]	L-iduronic acid / (L-iduronic acid + D-glucuronic acid)	Sulfur[c] / Hexosamine	D-Glucosamine residues with		
					unsubstituted amino groups[d]	sulfated amino groups[d]	acetylated amino groups[d]
	%	%				%	
Heparin	27.4	24.1[e]	0.67	2.56	5	65	30
Heparin, desulfated and N-acetylated[a]	32.6	24.8		0.31	<5	<5	>95
Heparin, desulfated and N-deacetylated[a]	35.1	29.2		0.33	90	0	15
Heparin, desulfated, N-deacetylated and N-sulfated[a]	31.8	31.1		1.52	5	85	15
Heparan sulfate	29.3	35.0[e]	0.15	0.50	<5	30	65
Heparan sulfate, N-deacetylated				0.49	50	25	20
Heparan sulfate, N-deacetylated and N-sulfated	34.4	30.3		1.18	20	55	30
Chondroitin sulfate	26.9	30.4[e]	<0.05[f]	0.61			
Dermatan sulfate	23.4	28.9[e]		1.18			
Hyaluronic acid	27.5	35.0		0			
[5-^{3}H]PS-NH$_3^+$			<0.05		55	<5	45
[5-^{3}H]PS-NH$_3^+$, N-deacetylated and N-sulfated					25	55	20
[5-^{3}H]PS-NSO$_3^-$			0.17[g]		<5	80	15
[5-^{3}H]PS-NSO$_3^-$, N-desulfated					60	25	15
[5-^{3}H]PS-NSO$_3^-$, N-desulfated and re-N-sulfated					20	65	15

[a] Heparin was 3 times desulfated, by treatment with methanol-HCl followed by deesterification of carboxyl methyl esters formed in the process (20). Part of the product was N-acetylated whereas another portion was N-deacetylated. Finally, part of the desulfated, N-deacetylated material was N-sulfated.

[b] Per cent of dry weight, not corrected for moisture nor for losses during hydrolysis.

[c] Molar ratios with hexosamine as 1.00.

[d] Per cent of total hexosamine. For experimental details, see Methods and the legend to Fig. 2. The effluent fractions from gel chromatography of deamination products were analyzed for uronic acid by the carbazole reaction (unlabeled polysaccharides) or for radioactivity (5-^{3}H-labeled polysaccharides). Of the labeled polysaccharides listed in the table, only [5-^{3}H]PS-NSO$_3^-$ and derivatives thereof contained L-iduronic acid (15 to 20% of the total uronic acid, as determined with a ^{14}C-labeled analog); since the 5-^{3}H label is lost during conversion of D-glucuronic to L-iduronic acid, the method used to determine N-substitution patterns may have given a slight systematic error with these samples. Another source of error is the occasional ring contraction which occurs during the deaminative cleavage (19); this results in an overestimation of N-acetylated residues.

[e] The ratio of glucosamine to total hexosamine was 1.0 for heparin and heparan sulfate and 0.0 for chondroitin sulfate and dermatan sulfate.

[f] The method employed to obtain uronic acid monosaccharides from polysaccharides has only been tested with heparin-like polymers (6).

[g] Determined by analysis of ^{14}C-labeled material.
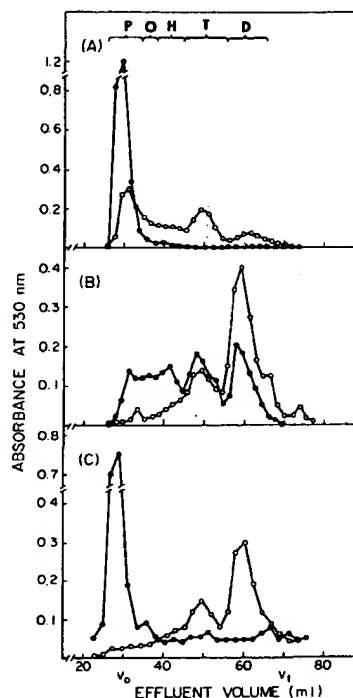

Fig. 2. Gel chromatography on Sephadex G-25 of products obtained by deamination of heparan sulfate (A), N-deacetylated heparan sulfate (B), and N-deacetylated and then N-sulfated heparan sulfate (C). Samples of 1.0 mg were treated with nitrous acid at pH 4.3 in a volume of 0.5 ml. After 10 min the reaction was interrupted by the addition of 1.25 ml of 1.1 M ammonium sulfamate, and the samples were applied to a column (1 x 94 cm) of Sephadex G-25, equilibrated with 1 M NaCl. The column was then eluted with 1 M NaCl at a rate of 3-4 ml per hour; fractions of 1.5-2.0 ml were collected and analyzed for uronic acid (● - ●) by the carbazole reaction. Another set of samples were deaminated at pH 4.3 as described above and were then desalted (by passage through a column of Sephadex G-15 equilibrated with 10% aqueous ethanol), concentrated and subjected to additional deamination, at pH 1.5, in 0.7 ml of the reagent described by Shively and Conrad (19). The products were again analyzed by gel chromatography on Sephadex G-25 (○ - ○).
The components of the effluent fractions were classified with regard to molecular size by comparison with the elution positions of reference oligosaccharides (6), as indicated in Frame A (P, polysaccharides; O, octasaccharides; H, hexasaccharides; T, tetrasaccharides; and D, disaccharides). The relative amounts of N-unsubstituted, N-sulfated and N-acetylated glucosamine residues in the parent polysaccharides were calculated from the distribution of deamination products between the various oligosaccharide classes; for further details, see Methods.

Biosynthesis of Heparin

PARTIAL PURIFICATION OF THE URONOSYL C-5 EPIMERASE*

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Heparosan *N*-sulfate D-glucuronosyl 5-epimerase, which catalyzes the conversion of β-D-glucuronosyl to α-L-iduronosyl residues in the course of heparin biosynthesis, has been purified approximately 9000-fold from the high speed supernatant fraction of a homogenate of a mouse mastocytoma. Following ammonium sulfate fractionation, the material precipitating between 35 and 60% saturation was subjected to a series of affinity chromatography steps on matrices containing immobilized concanavalin A, heparan sulfate, O-desulfated heparin, and Cibacron blue, respectively. Epimerase purified by this procedure yielded two major components on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme had approximately the same K_{av} as bovine serum albumin when chromatographed on Sepharose 6B.

The activity of the purified enzyme was increased 50-fold by addition of the fraction which was not adsorbed to concanavalin A-Sepharose. The stimulating factor is likely to be a protein since it was nondialyzable, heat labile, and lost activity on digestion with trypsin.

In a previous report (1), an assay was described for the glucuronosyl 5-epimerase which catalyzes conversion of polysaccharide-bound glucuronic acid to iduronic acid groups in the course of heparin biosynthesis. Some properties of the epimerase including its substrate specificity were also determined, with the microsome fraction from a homogenate of the Furth mastocytoma as a source of the enzyme. The present paper describes the purification of the epimerase from the soluble fraction of the tissue homogenate. Some additional properties of the enzyme are also reported, particularly the requirement for a protein factor which is necessary for full activity of the purified enzyme.

EXPERIMENTAL PROCEDURES

Materials

Bovine serum albumin, ovalbumin, transferrin, myoglobin, catalase, cytochrome c, ribonuclease A, and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes[1]) were purchased from Sigma. Trypsin, treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone, and soybean trypsin inhibitor were obtained from Worthington. Twice recrystallized papain was prepared according to Kimmel and Smith (4). Glucosamine and galactosamine were products of Pfanstiehl. Fluram (fluorescamine) was obtained from Hoffmann-La Roche. Con A-Sepharose, Sepharose 4B and 6B, and Sephadex G-200 were purchased from Pharmacia Fine Chemicals. Cibacron blue F3G-A was obtained from Polysciences. All other chemicals were of reagent grade and obtained from commercial sources.

Heparin (U. S., 155 units/mg) was obtained from Inolex, Pharmaceutical Division, Park Forest South, Ill. After desulfation, a portion of the product was *N*-acetylated, and another portion was deacetylated and re-*N*-sulfated. These procedures have been described previously (1).

Heparan sulfate from heparin by-products (Fraction 1.25:2 in Table VIII, Ref. 5) was a gift from Dr. J. A. Cifonelli, University of Chicago. This material was digested with papain in 0.01 M phosphate buffer, pH 7.4, containing 0.005 M EDTA, 0.005 M cysteine hydrochloride, and 0.5 M NaCl. After digestion, the polysaccharide was recovered by precipitation with cetylpyridinium chloride and converted to the sodium salt (5).

Mice of the strain LAF₁/J0305 were obtained from Jackson Laboratories, Bar Harbor, Maine.

Coupling of Heparan Sulfate, O-Desulfated Heparin, and Cibacron Blue F3G-A to Sepharose (Sephadex)

The purified heparan sulfate was coupled to Sepharose by the following procedure (6). Solid cyanogen bromide (1.8 g) was added to 50 ml of an aqueous solution of the polysaccharide (200 mg), the solution was cooled in ice, and 50 ml of Sepharose 4B, well rinsed in distilled water, was added all at once. The slurry was stirred in an ice bath, and the pH was raised to 11 with 5 M NaOH and maintained at this level for 10 min. The gel was then shaken gently at 4°C for 24 h, rinsed with 0.1 M ethanolamine, pH 8.5, and kept in this solution for 2 h to block remaining active sites. Finally, it was rinsed successively with 0.5 M NaHCO₃, water, 0.1 M acetate buffer, pH 5.0, 2 M NaCl, and water.

O-Desulfated heparin (200 mg), prepared from heparin by desulfation, deacetylation, and subsequent re-*N*-sulfation, was dissolved in 25 ml of water, the pH was adjusted to 4.5, and the solution was added to 50 ml of 1,6-diaminohexane-substituted Sepharose 4B (7), which had been extensively washed with water at pH 4.5. 0.5 g of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride was dis-

solved in 50 ml of water, the pH was adjusted to 4.5, and the solution was combined with the slurry under stirring. The pH of the mixture was readjusted to 4.5 every 15 min during a 2-h period. After 16 h, the gel was rinsed with 2 M NaCl, followed by water at pH 4.5. The amount of polysaccharide bound to the gel was estimated by hexosamine analysis on a Biocal 200 automatic amino acid analyzer. Packed gel (0.5 ml) was hydrolyzed in 3 ml of suprapure HCl under argon at 100°C for 3 h. The samples were evaporated, dissolved in 0.8 ml of starting buffer (0.2 M citrate, pH 2.2), and, after filtration, 0.5 ml was taken for analysis as described (8). The amounts of bound glucosamine were 0.8 and 1.6 μmol/ml for the gels containing heparan sulfate and O-desulfated heparin, respectively.

Cibacron blue F3G-A was coupled to Sephadex G-200 according to Böhme *et al.* (9).

Analytical Methods

Protein was estimated by fluorometric analysis essentially as described by Böhlen *et al.* (10), with bovine serum albumin as standard. To 250 μl of sample was added 50 μl of 0.6 M sodium borate, pH 8.5. During mixing on a Vortex mixer, 100 μl of fluorescamine reagent (0.3 mg of fluorescamine/ml of acetone) was added. Finally, 1 ml of 0.2 M sodium borate was added, and the relative fluorescence was measured with an Aminco spectrofluorimeter, model SPF 125. The excitation and emission wavelengths were 390 and 475 nm, respectively. When the amount of protein was limited, the volumes of sample and reagents were proportionately reduced to yield a final volume of 300 μl.

Polyacrylamide gel electrophoresis in SDS was carried out in standard gels (11.1 × 0.9) as described by Neville (11). The dimensions of the gels were 0.5 × 7 cm, and the lower buffer was 0.0308 M HCl, 0.4244 M Tris, pH 9.18. Before electrophoresis, enzyme solutions were dialyzed against 0.01% SDS in dialysis bags (6000 to 8000 molecular weight cut-off), which had been boiled in 0.5% EDTA, 1% NaHCO₃. The dialyzed samples were lyophilized and then dissolved in 50 μl of water. An equal volume of concentrated upper reservoir buffer which contained, in addition, 0.001 M EDTA, 10% 2-mercaptoethanol, 10% sucrose, 3.8% SDS was added, and the solutions were heated for 3 min at 100°C before application to the gels. After electrophoresis, the gels were removed from the tubes, and the dye front was marked by insertion of a surgical wire. The gels were fixed for 6 h in 50% methanol, 7.5% acetic acid and then stained overnight with Coomassie brilliant blue G 250 (0.25% dye in fixing solution). The gels were destained by diffusion and then rehydrated in 7.0% acetic acid.

Epimerase Assay

Substrate—Radioactively labeled microsomal heparin precursor polysaccharides were prepared by incubating microsomal enzyme with UDP-[5-³H]glucuronic acid, UDP-N-acetylglucosamine, and 3'-phosphoadenylyl sulfate as described (1). The product was used as substrate without fractionation by ion exchange chromatography and contained the four polysaccharide species, PS-NAc, PS-NH₃⁺, PS-NSO₃⁻, and PS-N/O-SO₃⁻ (see Fig. 1 in Ref. 1).

Reaction Conditions—Reaction mixtures contained, in a final volume of 300 μl, 1100 to 1500 cpm of substrate in 25 μl of water and 275 μl of enzyme in 0.05 M Hepes, 0.05 M KCl, 0.015 M EDTA, pH 7.4 (Buffer A). After incubation for 1 h at 37°C, the reaction was terminated by heating at 100°C for 2 min, and the samples were distilled as described (1). Aliquots (200 μl) of the distillates were mixed with 10 ml of Scintiverse (Fisher Scientific), and radioactivity was measured in a Packard scintillation counter, model 2450. Samples were counted for 20 min each.

Enzyme Purification

The source of 5-epimerase was the Furth mast cell tumor, which was propagated in LAF₁/J0305 mice by the following procedure. Mast cells were prepared by trypsinization of the tumor, and the cells were stored in Hanks' medium under liquid nitrogen until used (12). Before inoculation, the cells were thawed in a 37°C bath and diluted with Krebs-Ringer buffer to a final cell density of 8 × 10⁵/ml. One-hundred microliters of this suspension was inoculated into each hind leg of the mice, and tumors were allowed to develop for 17 days, at which time the animals were sacrificed by cervical dislocation and the tumors were removed, cooled on ice, and dissected free of muscular tissue. Purification of the epimerase was carried out at 4°C in the following manner and is summarized in Table I.

Step 1: Homogenization and Centrifugation—The tumors were minced and homogenized in a Potter-Elvehjem homogenizer in an equal volume of 0.1 M Tris, 0.3 M potassium phosphate, 0.003 M MgCl₂, 0.0025 M cysteine hydrochloride, pH 7.3. The homogenate was centrifuged at 20,000 × g for 10 min, and the supernatant liquid, which contained at least 50% of the total enzymatic activity, was collected and recentrifuged for 70 min at 97,000 × g. The high speed supernatant was stored at −20°C until sufficient material had been produced. Additional soluble enzyme could be obtained by re-extraction of the 20,000 × g pellet.

Step 2: Ammonium Sulfate Fractionation—(This step achieved only 1.5-fold purification. However, its omission interfered with subsequent purification and yielded less pure final product; therefore, it was routinely included.) To 700 ml of the supernatant liquid, solid ammonium sulfate was added under slow stirring to 35% saturation. Stirring was continued for 15 min after the salt had dissolved, and after another 15 min, the precipitate was collected by centrifugation at 35,000 × g for 1 h. The ammonium sulfate concentration was then brought to 60% saturation, and the precipitate was collected as before and was dissolved in 140 ml of 0.05 M Hepes, 0.05 M KCl, pH 7.4 (Buffer B).

Step 3: Chromatography on Concanavalin A-Sepharose—(In developing a procedure for the purification of heparosan N-sulfate D-glucuronosyl 5-epimerase, the ability of several potential affinity ligands to inhibit tritium release in the standard assay was first examined (Fig. 1). On the basis of this study, Sepharose-coupled

TABLE I
Effect of various salts on epimerase activity

All incubation mixtures contained 0.05 M Hepes, 0.05 M KCl, pH 7.4. Additions of salts were made to give the final concentrations indicated in the table.

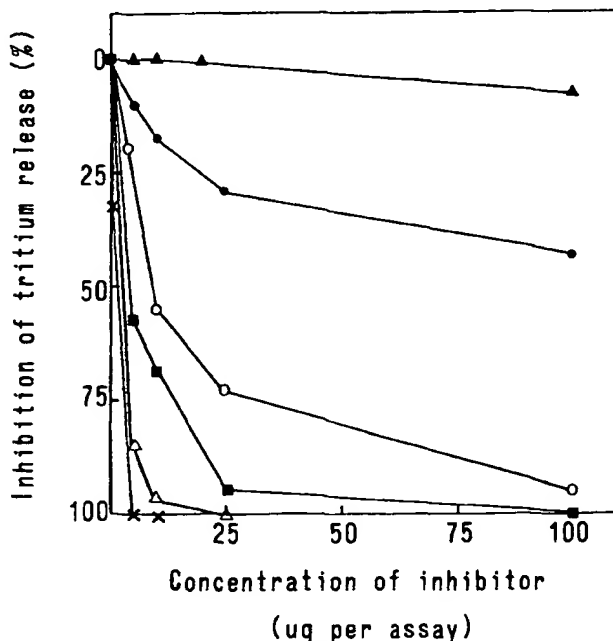| Salt added	Final concentration of added salt	Tritium liberated
	mM	*cpm*
None		3
EDTA	15	120
KCl	100	116
CaCl₂	25	30
MgCl₂	25	96
MnCl₂	25	9
Na₂SO₄	20	129
Na₂HPO₄	20	116

FIG. 1. **Effect of potential affinity ligands on epimerase activity.** Enzyme from Step 1 was incubated with tritium-labeled substrate in the presence of desulfated, acetylated heparin (▲——▲), heparan sulfate (●——●), heparin (○——○), O-desulfated heparin (■——■), concanavalin A (△——△), and Cibacron blue F3G-A (×——×).

inhibitory compounds, all of which retained epimerase, although to different extents, were selected for use in the purification procedure.)

The 35 to 60% ammonium sulfate fraction (140 ml) was diluted with 760 ml of Buffer B and applied to a column of Con A-Sepharose (2.1 × 15 cm), which had been equilibrated with the same buffer. Fractions of 22 ml were collected at a rate of 40 ml/h. The gel was subsequently washed with 5 bed volumes of 0.05 M Hepes, 0.25 M KCl, pH 7.4, and the enzyme was eluted at a rate of 20 ml/h with 250 ml of 0.05 M Hepes, 0.25 M KCl, 0.5 M methyl α-D-mannoside, pH 7.4 (Fig. 2).

Since the loss of activity in Step 3 was 79%, the possibility was considered that some factor required for full activity had been removed. When a reaction mixture containing Con A-Sepharose-purified enzyme was supplemented with nonadsorbed protein, the activity of the enzyme increased 3- to 4-fold. Calculated on the basis of this stimulated activity, the recovery in Step 3 was then 83%, with an overall recovery of 51%. Accordingly, enzyme activity was routinely assayed with and without "factor" for each step of the purification (Table I).

Step 4: Chromatography on Heparan Sulfate-Sepharose—The eluate from the preceding step was diluted with 1000 ml of 0.05 M Hepes, 0.01875 M EDTA, pH 7.4, and applied to a heparan sulfate-Sepharose column (2.1 × 16 cm), which had been equilibrated with Buffer A. After the matrix had been washed with Buffer A to remove nonadsorbed proteins, the epimerase was eluted with a linear salt gradient (mixing vessel, 250 ml of Buffer A; reservoir, 250 ml of 0.05 M Hepes, 0.25 M KCl, 0.015 M EDTA, pH 7.4). Fractions of 20 ml were collected at a rate of 40 ml/h and assayed for epimerase, protein, and conductivity (Fig. 3).

Step 5: Chromatography on Sepharose Coupled to O-Desulfated Heparin—The pooled active fractions (162 ml) eluted from the heparan sulfate-Sepharose column were diluted with 437 ml of 0.05 M Hepes, 0.015 M EDTA, pH 7.4 (Buffer C). This sample was applied to a column (1.5 × 15 cm) of Sepharose linked to O-desulfated heparin. After washing with Buffer A, the epimerase was eluted with a linear salt gradient as in Step 4, except that the volume in each vessel was 150 ml. Fractions of 19 ml were collected at a rate of 15 ml/h and assayed for epimerase activity.

Step 6: Chromatography on Cibacron Blue-Sephadex—Epimerase-containing fractions from Step 5 (132 ml) were diluted with Buffer C (225 ml) and applied to a column (2.5 × 6 cm) of Cibacron blue-Sephadex which had been equilibrated in Buffer A. After rinsing with 4 bed volumes of Buffer A, the enzyme was eluted with a linear salt
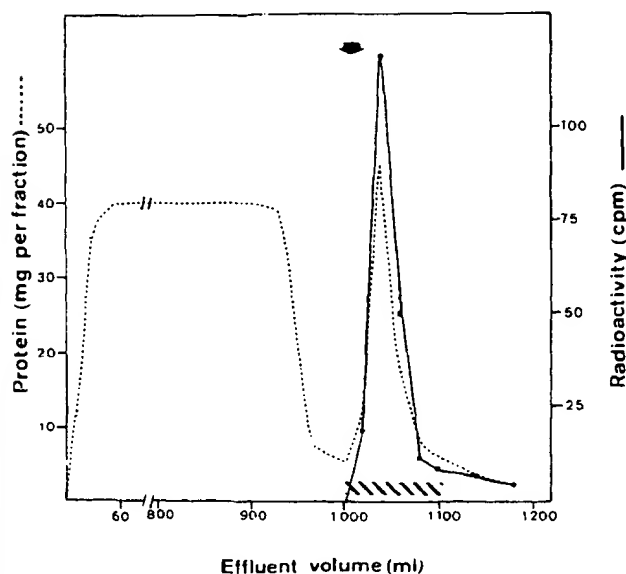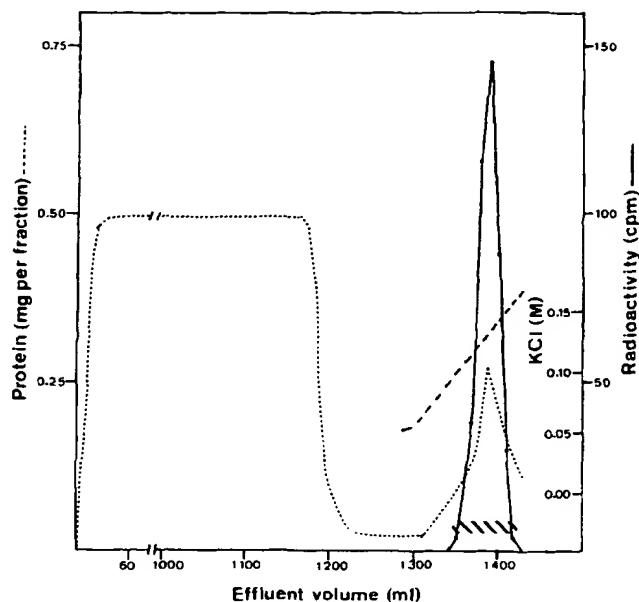

FIG. 3. **Purification of the uronosyl C-5-epimerase by affinity chromatography on heparan sulfate-Sepharose.** Epimerase from Step 3 was diluted and applied to the column (2.1 × 16 cm) and the retained enzyme was eluted by a linear gradient of potassium chloride (–––). Flow rate, 40 ml/h; fraction size, 20 ml. Enzyme activity (●——●) and protein (·····) were estimated. The fractions indicated by the *bar* were pooled and further purified.

gradient (mixing vessel, 200 ml of Buffer A; reservoir, 200 ml of 0.05 M Hepes, 0.5 M KCl, 0.015 M EDTA, pH 7.4). Fractions of 18 ml were collected at a rate of 15 ml/h. The fractions which contained epimerase activity (9 to 17) were pooled.

RESULTS AND DISCUSSION

Some Properties of Crude Epimerase—Prior to purification, some basic properties of the epimerase were determined, as observed in the standard assay system with the 97,000 × g supernatant fraction as enzyme source. The release of tritium was linear with time for slightly more than 1 h (Fig. 4), and the reaction rate increased with increasing enzyme concentration to a plateau value at approximately 1 mg of protein/ml (Fig. 5). The enzyme was active over a narrow pH range, with an optimum at pH 7.4 (Fig. 6). Particularly important from a practical point of view was the marked dependence on ionic strength. As seen in Table I, the enzyme had negligible activity in 0.05 M Hepes, 0.05 M KCl, pH 7.4, but addition of EDTA to a final concentration of 0.015 M (standard buffer) yielded maximal tritium release. Similar results were obtained when the KCl concentration was increased to 0.1 M or upon addition of other salts (Na$_2$SO$_4$, Na$_2$HPO$_4$, and MgCl$_2$) at appropriately chosen concentrations. Only moderate stimulation was observed with CaCl$_2$, and MnCl$_2$ was comparatively ineffective. In agreement with previous studies of the microsomal epimerase (1), it is concluded that a specific metal ion requirement does not exist. However, exact control of ionic strength is necessary for reproducible assay of epimerase activity since the enzyme is active only over a narrow ionic strength range. The failure of manganous ion to stimulate activity is not presently understood, but this observation should be contrasted with the finding that the uronosyl 5-epimerase involved in dermatan sulfate biosynthesis requires manganous ion for full activity (13).

A K_m of 5.5 × 10^{-9} M was determined for the substrate used in this study. This value is subject to upward revision since the amount of endogenous, unlabeled substrate in the prepa-

FIG. 2. **Purification of uronosyl C-5-epimerase by affinity chromatography on concanavalin A-Sepharose.** The ammonium sulfate fraction was diluted and applied to the column (2.1 × 15 cm) and the retained enzyme was eluted with 0.05 M Hepes, 0.25 M KCl, 0.5 M methyl α-D-mannoside, pH 7.4. The *arrow* indicates the start of the elution. Flow rate, 40 ml/h; fraction size, 22 ml. Enzyme activity (●——●) and protein (–––) were measured. The fractions indicated by the *bar* were pooled and further purified.
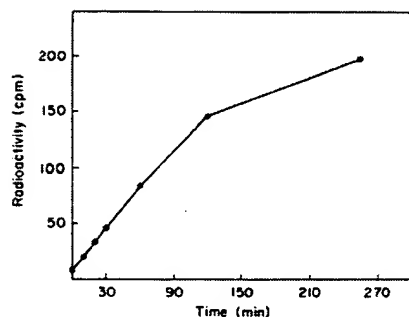
FIG. 4 (*left*). **Time course of tritium liberation.** Seventy micrograms of protein from the 97,000 × *g* supernatant fraction was incubated with substrate (2 × 10³ cpm) for the indicated periods of time.

FIG. 5 (*left center*). **Effect of concentration of enzyme (Step 1) on tritium release.**

FIG. 6 (*right center*). **Effect of pH on epimerase activity.** Enzyme from Step 1 was diluted 200-fold with 0.05 M Hepes, 0.05 M KCl, 0.015 M EDTA of the appropriate pH.
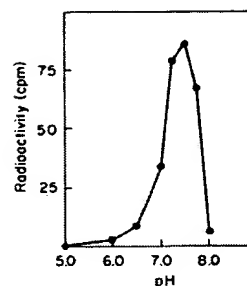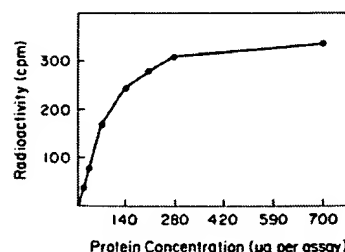
FIG. 7 (*right*). **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the most purified epimerase fraction.** The enzyme obtained in Step 6 was concentrated, desalted, treated with SDS, as described in the text, and subjected to gel electrophoresis. The band in the bottom part of the gel is a surgical wire, which indicates the buffer front. Molecular weight markers (bovine serum albumin, ovalbumin, and myoglobin) were electrophoresed on a separate gel, and approximate molecular weights for the two components of the enzyme preparation were calculated as described (11).

TABLE II

Purification of uronosyl C-5 epimerase

Enzyme fraction	Total volume	Total protein	Total activity		Specific activity		Recovery		Purification	
			Without factor	With[a] factor	Without factor	With[a] factor	Without factor	With[a] factor	Without factor	With[a] factor
	ml	*mg*	*cpm*		*cpm/mg protein*		*%*		*−fold*	
1. 97,000 × *g* supernatant	700	7,000	39.2 × 10⁶	55.3 × 10⁶	5,600	7,900	100	100	1.0	1.0
2. Ammonium sulfate fraction	140	3,250	27.2 × 10⁶	34.2 × 10⁶	8,374	10,530	69	62	1.5	1.3
3. Eluate from Con A-Sepharose	250	270	5.7 × 10⁶	28.4 × 10⁶	21,100	105,000	15	51	3.8	13.3
4. Eluate from heparan sulfate-Sepharose	162	4.89	816,000	16.2 × 10⁶	166,900	3,313,000	2.1	29	29.8	419
5. Eluate from Sepharose-linked *O*-desulfated heparin	132	0.92	242,000	14.2 × 10⁶	263,000	15,430,000	0.6	26	47	1,950
6. Eluate from Cibacron blue-Sephadex	171	0.21	297,000	14.0 × 10⁶	1,414,000	66,670,000	0.8	25	253	8,440 (8,660)[b]

[a] One-hundred microliters of the protein fraction, which was not retained by Con A-Sepharose in Step 3, was added to these incubations. This amount of factor gave maximal stimulation.

[b] Calculated on the basis of numbers which have not been rounded off.

ration was not known and could not be readily quantitated. It should also be pointed out that the substrate preparation was a mixture of several polysaccharide species and that only about one-third of the material had the proper substrate structure, as indicated by the finding that no more than 30% of the label was released as ³H₂O upon exhaustive incubation with the epimerase.

Properties of Purified Epimerase—As seen from Table II, the most highly purified preparation of heparosan *N*-sulfate D-glucuronosyl 5-epimerase was obtained in an overall yield of 25% and purified approximately 8700-fold to a specific activity of 67 × 10⁶ cpm/mg of protein. Enzyme of similar specific activity has been obtained in three separate preparations.

On SDS-polyacrylamide gel electrophoresis, enzyme from Step 6 gave a broad band (possibly representing two overlapping components) with a migration rate corresponding to a molecular weight of 54,000. In the presence of 2-mercaptoeth-

anol, two major components were observed which migrated at rates corresponding to molecular weights of 52,000 and 56,000, respectively (Fig. 7). Several extremely faint bands were also visible; one of these was observed in a blank run in which dialyzed buffer was analyzed by the same procedure. It has not been possible to establish whether one or both of the two major components are identical with the enzyme since no enzymatic activity could be detected in dialyzed extracts of the gel. No activity was detectable in the gel after electrophoresis in the absence of SDS.

On analysis of the purified enzyme (Step 5 or Step 6) by gel chromatography on Sepharose 6B (for details, see Ref. 14), the activity emerged as a symmetrical peak slightly behind bovine serum albumin. This elution position is consistent with the molecular weights of 52,000 and 56,000 calculated for the major components observed on SDS-polyacrylamide gel electrophoresis. It cannot be presently ruled out, however, that the fully active enzyme consists of smaller subunits which
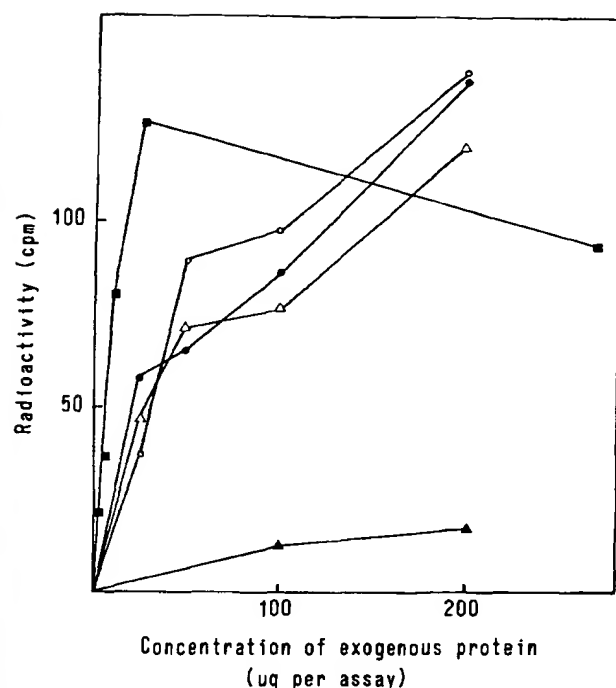
FIG. 8. **Effect on epimerase activity of the addition of increasing amounts of extraneous proteins to a standard incubation mixture.** Factor preparation (■——■), cytochrome *c* (●——●), catalase (△——△), ribonuclease A (○——○), and bovine serum albumin (▲——▲) were added to a standard incubation mixture containing enzyme obtained in Step 6. The factor preparation consisted of protein not adsorbed to Con A-Sepharose in Step 3.
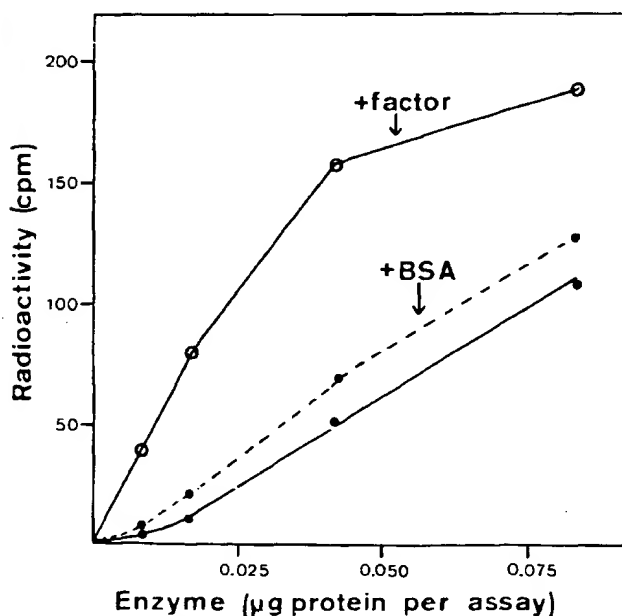

FIG. 9. **Relationship between enzyme concentration and activity in the absence (●——●) of added protein and in the presence of 100 μg of factor (○——○) or 500 μg of bovine serum albumin (●- - -●).** Enzyme was obtained from Step 5, and the factor preparation consisted of protein not adsorbed to Con A-Sepharose in Step 3. Similar results were obtained when ovalbumin and transferrin were substituted for bovine serum albumin.

would migrate to the positions of the trace components found on SDS-polyacrylamide gel electrophoresis.

An interesting discrepancy should be pointed out which concerns the molecular weight of the epimerase at various

stages of purification. Whereas the highly purified enzyme was eluted near bovine serum albumin (molecular weight, 68,000), crude enzyme (Step 2) gave a single peak of activity which emerged shortly after the void volume and well ahead of xylosyltransferase from embryonic chick cartilage (molecular weight, 95,000 to 100,000) when analyzed by chromatography on Sephadex G-200 (for details, see Ref. 14). This suggests that the epimerase may associate with other proteins present in the early stages of purification.

Properties of the Stimulating Factor—By recombining enzyme fractions with the protein fraction not adsorbed to Con A-Sepharose in Step 3, a 3- to 5-fold stimulation of epimerase activity was obtained. The degree of stimulation was related to the purity of the enzyme and was as high as 50- to 60-fold for the most active preparations (Table II). At low concentrations of highly purified enzyme, little or no activity was observed in the absence of factor (Figs. 8 and 9).

The nature of the stimulating effect has not yet been determined, nor is the identity of the factor known. However, the activator is likely to be a protein since it was nondialyzable and was destroyed by heating at 60°C for 30 min (Fig. 10) or by digestion with trypsin (Table III). In contrast to the
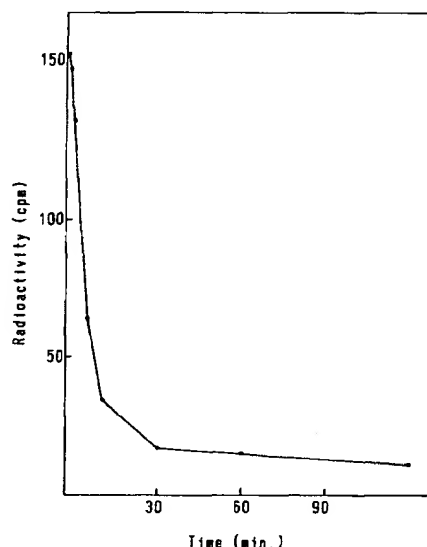

FIG. 10. **Heat inactivation of crude factor.** Protein not adsorbed to Con A-Sepharose in Step 3 was kept in a bath at 80°C until the temperature of the solution reached 60°C. It was then transferred to a water bath at 60°C, and at the indicated times, a sample was added to a standard incubation mixture containing enzyme obtained in Step 6. The zero time value was obtained with untreated factor, and the second value was obtained with factor that was removed when the temperature had reached 60°C.

TABLE III

Effect of trypsin digestion on factor activity

One milligram of factor preparation was digested with 1 mg of trypsin at 37°C in 1 ml of Buffer A. After 1 h, 1 mg of soybean trypsin inhibitor was added, and the digest (100 μl) was assayed for stimulating activity with enzyme from Step 4. The same result was obtained when the trypsin digestion was interrupted by addition of diisopropyl fluorophosphate to a final concentration of 2 mM.

Addition	Activity
	cpm
None	19
Factor, 100 μl[a]	93
Digested factor, 100 μl	39
Trypsin·soybean trypsin inhibitor complex, 100 μg	43

[a] Incubation of the factor preparation for 2 h at 37°C did not diminish stimulating activity. Trypsin·soybean trypsin inhibitor complex did not increase the stimulating activity of the factor preparation.

epimerase, the factor did not lose activity on exposure to 4 M guanidinium chloride (followed by dialysis against Buffer A).

Since the stimulation might be nonspecific, a number of proteins were tested for their effect on purified epimerase from Step 6. As seen in Fig. 8, ribonuclease A, cytochrome *c*, and catalase were capable of stimulating epimerase activity to the same extent as the unknown factor, but only at much higher concentrations. Other proteins, such as bovine serum albumin (Figs. 8 and 9), ovalbumin, and transferrin (data not shown), had only slight stimulatory effect. From a qualitative point of view, these experiments would seem to support a nonspecific mechanism of stimulation caused by certain active structures present in some proteins but not in others. On the other hand, the high potency of the crude factor preparation, which consists of a multitude of proteins, rather suggests that a single compound with a specific function is responsible for the observed effect. However, attempts to isolate the active principle by gel chromatography and ion exchange chromatography have so far been unsuccessful.

Some kinetic aspects of the stimulation by factor deserve further comment. As shown in Fig. 9, a plot of epimerase activity *versus* enzyme concentration was not linear at low concentrations, but addition of factor resulted in a linear relationship. This was not due to protection of the enzyme by factor since linearity with time was observed even at the lowest enzyme concentrations. It should also be noted that the stimulatory effect was highest at low concentrations of enzyme. In the absence of knowledge of the mechanism of epimerase action, these observations cannot be properly interpreted at this time.

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